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GRANT NUMBER: DAMD17-94-J-4229

TITLE: Regulation of Breast Cancer Invasion and Metastasis by Progestin and Antiprogestin

PRINCIPAL INVESTIGATOR: Yuenian E. Shi, Ph.D.

CONTRACTING ORGANIZATION: Long Island Jewish Medical Center
New Hyde Park, New York 11042

REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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# REPORT DOCUMENTATION PAGE

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| 1. AGENCY USE ONLY (Leave blank) | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
|  | October 1995 | Annual 26 Sep 94 – 25 Sep 95 |

**4. TITLE AND SUBTITLE**
Regulation of Breast Cancer Invasion and Metastasis by Progestin and Antiprogestin

**5. FUNDING NUMBERS**
DAMD17-94-J-4229

**6. AUTHOR(S)**
Yuenian E. Shi, Ph. D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
Long Island Jewish Medical Center

New Hyde Park, New York 11042

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING AGENCY REPORT NUMBER**

**11. SUPPLEMENTARY NOTES**

**12a. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for public release; distribution unlimited

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**13. ABSTRACT** (Maximum 200 words)

It is known that breast cancer is dependent upon estrogenic hormones in about one-third of all clinical cases and can be inhibited by antiestrogenic antagonists. We are now **hypothesizing** that progestational hormones may also function as a stimulus for onset and progression of breast cancer and antiprogestin can interrupt these processes. The overall goals are to develop an *in vivo* model system for evaluating effects of progestins and antiprogestins on human breast tumor growth and metastasis. Using our recently established T47D-derived TKS-7 (FGF-4 transfected) metastatic model, we have initiated experiments designed to investigate the effects of progestational hormones and antiprogestin onapristone on the regulation of human breast cancer growth and metastasis in nude mice. In addition, we will attempt to identify mechanisms underlying these regulations. TKS-7 behaves like a hormone-independent but still hormone responsive phenotype in nude mouse. Progesterone significantly stimulates the tumor growth; and this progesterone-induced growth stimulation can be inhibited by antiprogestin onapristone. Furthermore antiestrogen tomaxifen treatment enhances the progestational stimulation of tumor growth.

**14. SUBJECT TERMS**
Invasion and Metastasis, Progestin and Antiprogestin, Angiogenesis, Fibroblast Growth Factor, 67KDA Laminin Receptor, Integrin, Breast Cancer, FGF-4

**15. NUMBER OF PAGES**
14

**16. PRICE CODE**

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
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## BACKGROUND

Regulation of growth and development of mammary gland involves a balance between the actions of the two major female sex steroid hormones, estradiol and progesterone. It is known that breast cancer is dependent upon estrogenic hormones in about one-third of clinical cases and can be inhibited by antiestrogenic antagonists [1]. From an endocrinological aspect, the view that estrogen are the major adverse hormonal factor in onset and progression of human breast cancer has dominated thinking in this area [2]. Conversely, the other ovarian steroid, progesterone, and its synthetic derivatives were initially thought to be protective, a view largely based on their differentiating and antiproliferative effects on endometrium [3,4]. However There is a growing body of evidence suggests that the mechanisms by which estrogen and progesterone regulate the proliferation and differentiation of uterine epithelial cells may not apply equally to the breast [5].

In **summary** of recent *in vitro* and *in vivo* studies suggest that progesterone may be more important than estrogen as an ovarian stimulus in driving proliferation of normal human and rodent breast epithelium [6-9]. One might expect that some aspects of these complex progestin-regulated events might be **retained** in breast cancers. In fact, the **more recent** experiments have demonstrated that progestational hormones stimulate the growth of carcinogen-induced and transplantable rat mammary tumors [10-11], and spontaneous mouse mammary tumors [12]. These observations could explain the increasing number of reports [13-14] connecting the pill use in young women with increased risk of breast cancer, and particular the very recent report on the **New England Journal of Medicine** showing that the risk of breast cancer was significantly increased among women who were currently using estrogen alone or estrogen plus progestin or progestin alone as compared with postmenopausal women who had never used hormones [15]. Therefore, progesterone has been suggested as a stimulus for the growth of human breast tumors. A major hurdle for fully testing this hypothesis has been the creation of human model systems whereby progestin-sensitive human cell lines are grown as tumors in the nude mouse. We are currently proposing to develop a T47D-derived and progestin-sensitive model(s) for evaluating progestational and antiprogestational regulation of breast cancer growth and progression.

## PRELIMINARY RESULTS

**<u>Effects of progestational hormones and antiprogestin on TKS-7 tumor growth</u>** We have selected TKS-7 cells for our hormonal regulation studies. We injected 5 millions cells into mammary fat pat of ovariectomized athymic nude mice at the age of 6 weeks. Two inoculations were injected for each mouse; 5 mice per group. We treated the mice with a slow time-releasing pellet containing either 1.5 mg three-week releasing medroxyprogesterone acetate (MPA) or 0.7 mg sixty-day releasing estradiol (Innovative). For that purpose, the mice were implanted s.c. in the intrascapular region 1 day before injection of cells. MPA pellets were replaced every 3 weeks. Tumor size was determined at intervals by measurement of two right angle diameters with a caliper. The mice were autopsied at the end of six weeks, and tumors and organs were processed and analyzed. As demonstrated in **Fig. 1** from two experiments, TKS-7 behaviors like a hormone-independent but still hormone responsive cell line. While estrogen is still a major driving force for TKS-7 tumor growth, inducing a 16-fold increase over control and a 100% tumor incidence, progestin MPA

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stimulates the tumorigenesis 2.5 fold over control; the tumor incidence was increased from 60% of control group to 90% of MPA-treated group.

Vector alone transfeced parental T47D cells were also examined for their responses to hormones. As we previously reported [16], parental T47D cells were poorly tumorigenic in both control and MPA-treated mice, no T47D tumors were observed. In mice implanted with the estradiol pellets, T47D cells formed small non-progressive tumors that subsequently regressed after 4 weeks (data not shown).

Several reports have demonstrated that synthetic progestins exert some estrogenic activity [17-19]. This raises the concern that the MPA-induced TKS-7 tumor growth may be mediated by a weak estrogenic activity but not by progestational activity. In order to rule out this possibility, we have began to use progesterone instead of progestin. In the following experiments, a 10 mg three-week releasing progesterone pellet was inoculated subcutaneously. The TKS-7 tumors showed a slow growth rate in first three weeks and gradually increased. The experiment was terminated at 8 weeks, by which time the size difference between control and progesterone-treated tumors was highly significant. As we anticipated, like MPA, progesterone stimulates the tumorigenesis 5 fold over control; the tumor incidence was also increased from 50% of control group to 70% of progesterone-treated group (**Fig. 2**).

We have obtained a newly developed antiprogestin onapristone from Dr. Henderson at Schering AG Berlin. This new antiprogestin onapristone (ZK98,299), a derivative of RU486, has high antiprogestin activity but low antiglucocorticoid activity [20]. Because of its antitumor activity in experimental mammary tumor models, we have explored its potential therapeutic value against breast cancer in our TKS-7 hormone-responsive human breast tumor model.

We have requested a special order of a slow-releasing onapristone pellets (5 mg/pellet, three week releasing) from Innovative, Inc (Ohio). The dose of onapristone was initially used at 5 mg/21 days, or 0.238 mg/mouse/day or 11.9 mg/kg/day (nude mouse is about 20g), which is compatible to previously published doses for rat and mouse models [21-23]. As demonstrated in **Fig. 2**, onapristone alone did not change the basal levels of tumorigenic activities of TKS-7 cells as measured by tumor size and tumor incidence. However, when the antiprogestin pellet was inoculated to progesterone-treated mice, onapristone significantly inhibits the progesterone-induced tumor growth. The tumor size was reduced from 210 $mm^3$ in progesterone-treated group to 77 $mm^3$ in progesterone- and onapristone-treated group (64% inhibition); no significant change of tumor incidence was observed. Lymph node metastasis were also examined by histological analysis. No lymph node metastasis was observed in control mice. However, we identified four lymph node metastases in progesterone-treated mice. A representative picture of such lymph node metastasis was shown in **Fig. 3**. Furthermore, onapristone treatment resulted in a reduction of progesterone-induced lymph node metastasis (**Fig. 2**). These results are consistent with the previously established metastatic MCF-7 cells showing that the rate of metastasis depends upon time since inoculation and tumor size [24,25].

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We are also interested in the effects of antiprogestin alone compared to its effects in combination with antiestrogen in an attempt to test the new endocrine therapy in the tamoxifen relapse setting. The rationale behind this antiestrogen-antiprogestin interaction derives from our assumption that antiestrogen tamoxifen, also a partial estrogen agonist, may increase PR expression in breast cancer cells and therefore enhance their responses to progesterone and onapristone. To test this hypothesis, we first settled out a experiment to investigate the interactions between tamoxifen and progesterone in our TKS-7 model. As demonstrated in **Fig. 4**, a **synergistic** effects of tamoxifen and progesterone was observed in TKS-7 tumor growth. Progesterone alone stimulated tumor growth 4-fold over control; tamoxifen also stimulated tumor growth (2.5-fold), although to a less extent as compared to progesterone, presumably due to its estrogenic activity. When the mice were treated with a combination of tamoxifen and progesterone, the resulting TKS-7 tumors were significantly larger than that of tamoxifen alone or progesterone alone. The tumor size was increased from 83.6 mm$^3$ in tamoxifen-treated group and 134 mm$^3$ in progesterone-treated group to 259 mm$^3$ in the combination group. Picture in **Fig. 5** shows the tumors from control, progesterone-treated, tamoxifen-treated and progesterone/tamoxifen-treated mice. Such synergistic interaction between tamoxifen and progesterone on TKS-7 tumor growth may due to the tamoxifen-mediated estrogenic effect on up-regulation of PR. We anticipate that the PR levels in tamoxifen-treated mice are increased as compared to that of non-treated or progesterone-treated mice.

To our knowledge, this is the first report on the in vivo stimulation of human breast tumor growth and lymph node metastasis by progestational hormones and the inhibition by antiprogestin onapristone. This information is extremely important in terms of evaluation of consequences of progestin use in contraception, postmenopausal replacement and breast cancer hormone therapy. We are currently in preparation of a manuscript.

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# Fig. 1. Hormonal Regulation of TKS-7 Tumor Growth
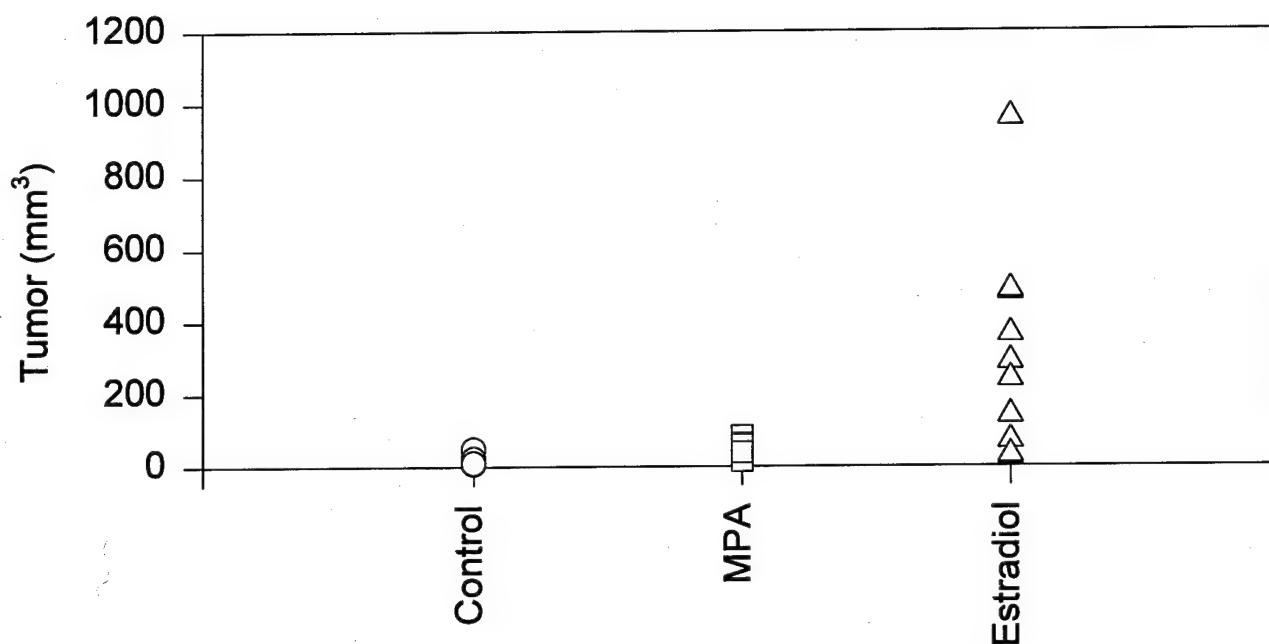


Effects of MPA and estradiol on TKS-7 tumor growth

|  | Tumor size (mm³ + SE) | Tumor incidence (%) |
|---|---|---|
| Control | 18.8+7.7 | 50 |
| MPA | 47.1+15.7 | 90 |
| Estradiol | 308.2+90.5 | 100 |

5 millions cells were injected into mammary fat pad of ovariectomized nude mice of age 6 weeks. 2 injections for each mouse; and 5 mice for each group. MPA, 1.5 mg/pellet/21 days; estradiol, 0.72 mg/pellet/60 days. The treatments with MPA were replaced every three weeks. Tumors were harvested after 6 weeks. Tumor incidences were calculated based on the number of tumors at the time of termination over the total 10 injections.
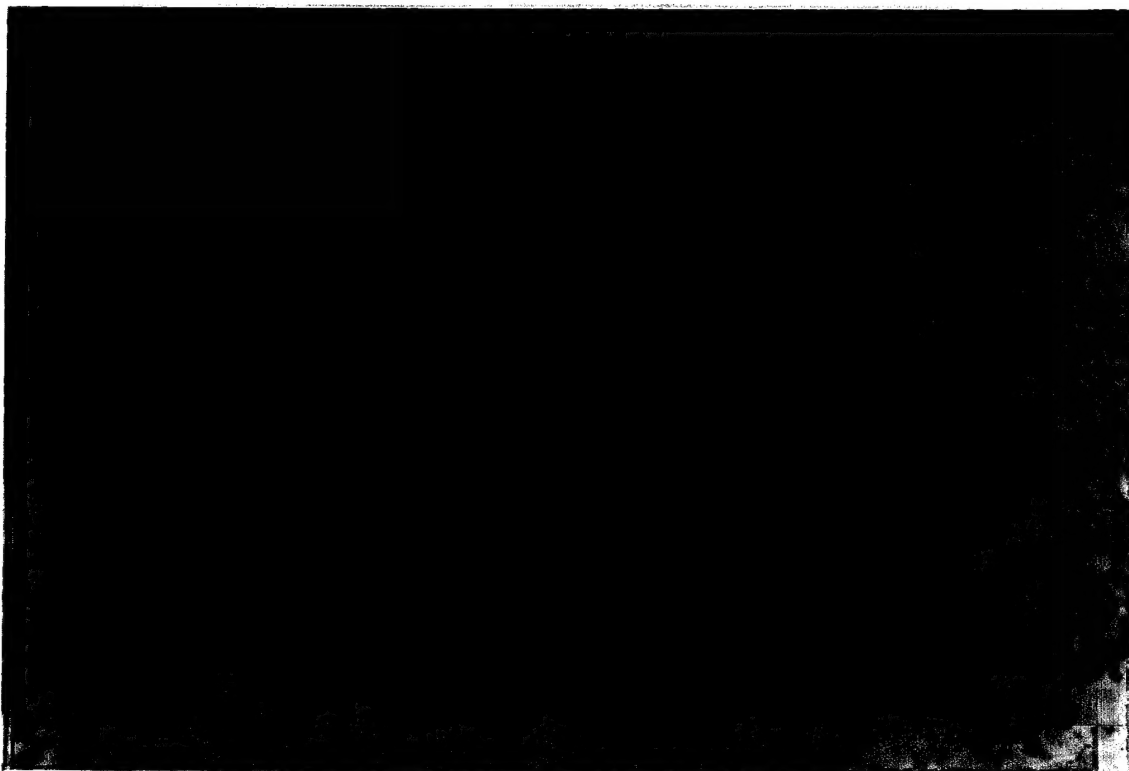
# Fig. 2. Effects of progesterone and onapristone on TKS-7 tumor
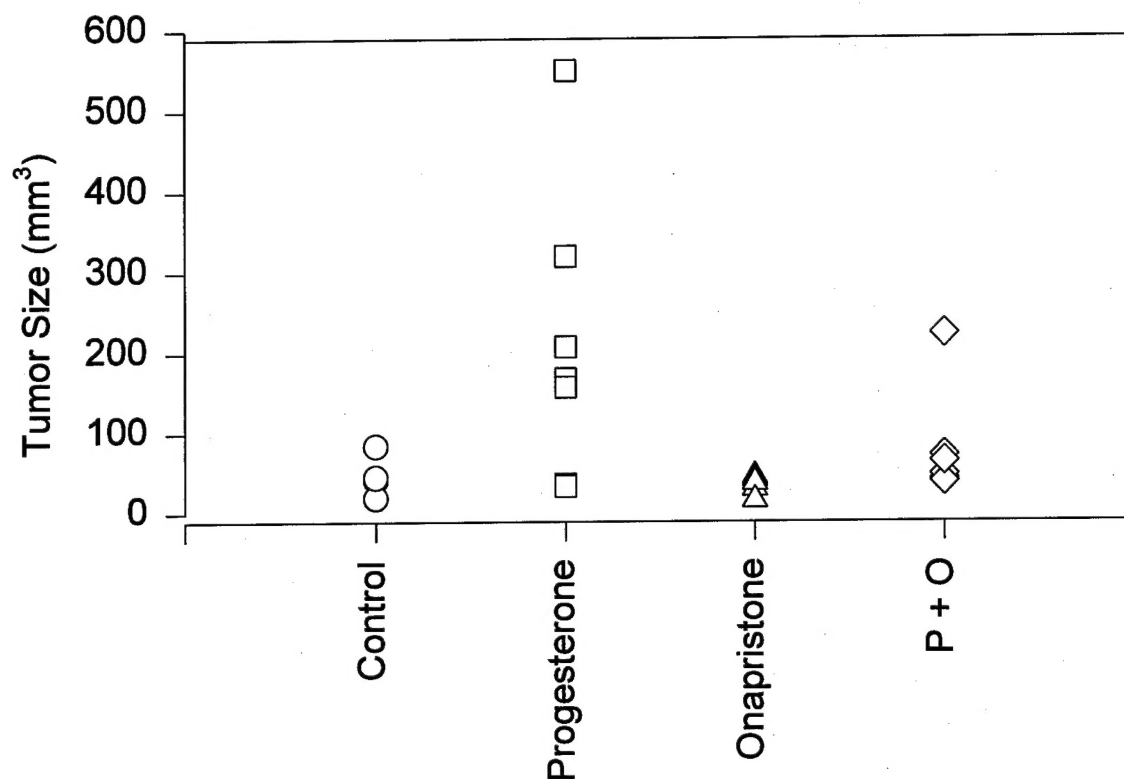


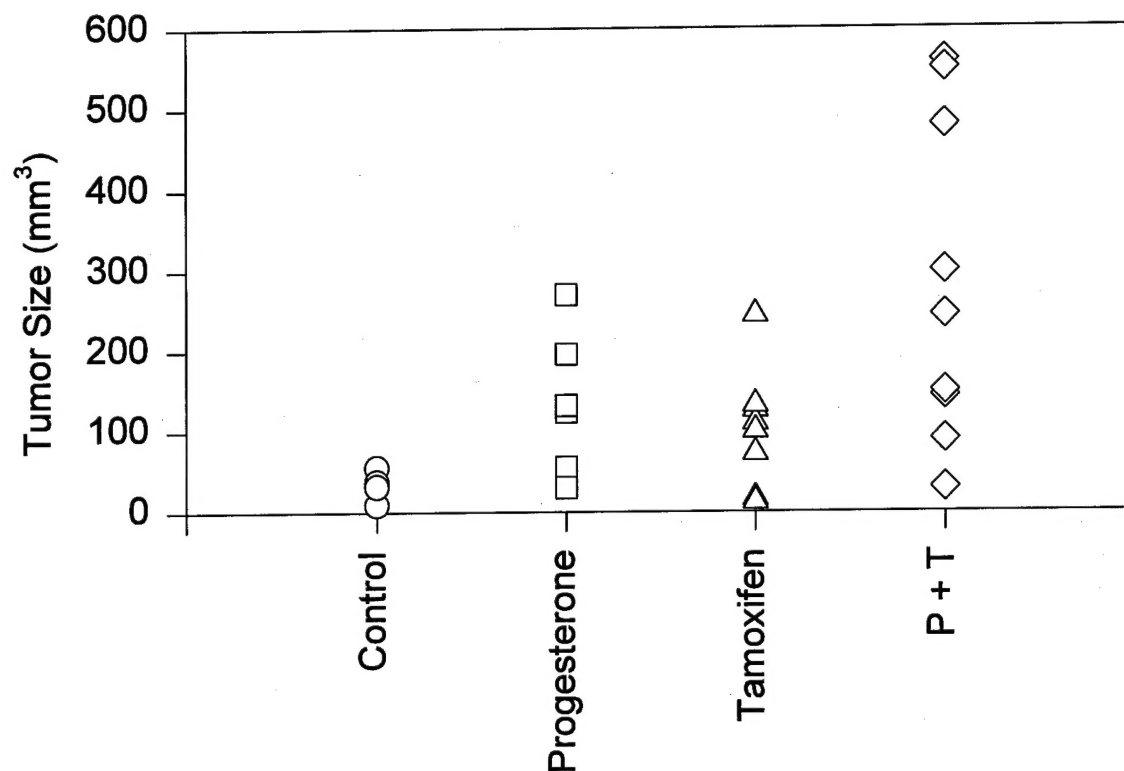Effects of progesterone and onapristone on TKS-7 tumor growth

|  | Tumor size (mm$^3$ + SD) | Tumor incidence (%) | Lymph node metastasis |
|---|---|---|---|
| Control | 41.9+11.8 | 50 | 0 |
| Progesterone (P) | 210.5+67.9 | 70 | 4 |
| Onapristone (O) | 33.4+4.3 | 60 | 0 |
| P+O | 77+24.9 | 70 | 1 |

5 millions cells were injected into mammary fat pad of ovariectomized nude mice of age 6 weeks. 2 injections for each mouse; and 5 mice for each group. Progesterone, 10 mg/pellet/21 days; onapristone, 5 mg/pellet/21 days. The treatments with progesterone and onapristone were replaced every three weeks. Tumors were harvested after 8 weeks. Tumor incidences were calculated based on the number of tumors at the time of termination over the total 10 injections. Lymph nodes near the cell injection sites were processed for histological analysis. Invasion of TKS-7 breast cancer cells to lymph node was clearly seen in four tumor injections in progesterone-treated mice. No lymph node metastasis were observed in either control or onapristone-treated mice; and only one lymph node metastasis was identified in progesterone- and onapristone-treated mice. The lymph node metastasis was defined by the appearance of the small island or foci of breast cancer cells which are bigger and lighter (hematoxylin stain) than the lymphatic cells

**Fig. 3**. Evolution of histology of ipsilateral lymph node metastasis from progesterone-treated tumor. 5 millions TKS-7 cells were injected into the mammary fat pad of the ovariectomized mice that were treated with progesterone (10mg/21 days). Animals were scarified 8 weeks after inoculation, and the tumors and lymph nodes were processed for analysis. ➡: tumor; ▲: lymph node.

# Fig. 4. Effects of progesterone and tamoxifen on TKS-7 tumor growth



Effects of progesterone and tamoxifen on TKS-7 tumor growth

|  | Tumor size (mm³ + SD) | Tumor incidence (%) |
| --- | --- | --- |
| Control | 33.4+16.7 | 40 |
| Progesterone (P) | 134.5+36.3 | 70 |
| Tamoxifen (T) | 83.6+23.8 | 100 |
| P+T | 259+65.9 | 100 |

5 millions cells were injected into mammary fat pad of ovariectomized nude mice of age 6 weeks. 2 injections for each mouse; and 5 mice for each group. Progesterone, 10 mg/pellet/21 days; tamoxifen, 5 mg/pellet/60 days. The treatments with progesterone were replaced every three weeks. Tumors were harvested after 6 weeks. Tumor incidences were calculated based on the number of tumors at the time of termination over the total 10 injections.
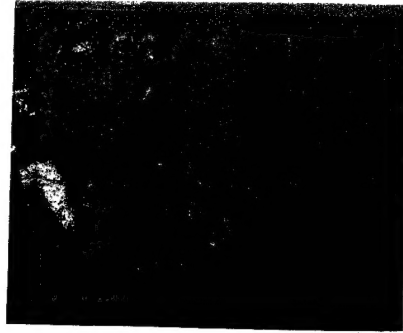
**Fig. 5.** Regulation of TKS-7 tumor growth by steroid hormones. 5 millions cells were injected into the mammary fat pad of the ovariectomized nude mice. The mice were divided into four groups: control (first line); progesterone-treated, 10 mg/pellet/21 days (second line); tamoxifen-treated, 5 mg/pellet/60 days (third line); and progesterone- and tamoxifen-treated (fourth line). Tumors were harvested after 6 weeks. 5 representing tumors from treated groups and 4 representing tumors from control group were placed on the dryice. Although both progesterone and tomaxifen stimulated tumor growth, combined treatment gave the biggest stimulation. Please **notice** that the differences of the real tumor sizes among the different groups are more dramatic than it looks like in the picture because the tumor sizes in the picture only reflect the 2-D measurement.